FED-BATCH CONTROL BASED UPON THE MEASUREMENT OF INTRACELLULAR NADH

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INTRODUCTION

This paper describes a series of experiments demonstrating that on-line measurements of intracellular NADH by culture fluorescence can be used to monitor and control fermentation processes. A distinct advantage of intracellular NADH measurements over other monitoring techniques such as pH and dissolved oxygen is that it directly measures real-time events occurring within the cell rather than changes in the environment and when coupled with other measurement parameters can provide a finer degree of sophistication in process control.

Culture fluorescence measurement techniques were first introduced by Duysens and Amesz.[1] They noted that the fluorescence spectrum for suspensions of aerobic baker's yeast was similar to the one for NADH and that the fluorescence signal could be significantly enhanced by additions of ethanol or glucose. Damped oscillations in fluorescence were observed after the establishment of anaerobic conditions. These oscillations were similar to those reported earlier by Chance[2] in yeast using absorption spectrophotometry. Chance and Thorell[3] found that most cellular fluorescence in eucaryotic cells was localized within mitochondria where NADH is concentrated because of its role in respiration. Chance et. al.[4] investigated the initiation of fluorescence oscillations in suspensions of starved yeast. The oscillations are caused by the sudden rates of glycolysis (Pasteur Effect). This work established a laboratory technique to follow intracellular metabolism.

Harrison and Chance[5] later built an instrument capable of measuring culture fluorescence within a fermentor. With this system, they were able to monitor aerobic/anaerobic transitions in continuous cultures of Klebsiella aerogenes. Using a similar device, Zabriskie and Humphrey[6] established a linear relationship between the log of culture fluorescence and the log of cell concentration. Ristroph et. at.[7] studied the relationship between culture fluorescence and the growth of Candida utilis in a fed-batch fermentation during which ethanol was added in discrete pulses. In this process, excessive ethanol feeding rates cause acetate to accumulate as a by-product. In the absence of ethanol, the yeast can assimilate the accumulated acetate. After each ethanol pulse, culture fluorescence peaks and declines gradually

as the ethanol is consumed. At the point where ethanol is exhausted and acetate consumption begins, the fluorescence declines more rapidly. Corresponding changes in the respiratory quotient indicate the metabolic changes accompanying the diphastic growth. The reproducible response of culture fluorescence to ethanol and acetate metabolism suggests that the instrument could be used to control the ethanol feeding schedule to improve process yield and productivity.

NADH IN METABOLISM

A highly simplified summary of basic cellular metabolism, illustrated in figure 7-1, shows how NAD+ is constantly cycled between the oxidized form (NAD+) and the reduced form (NADH). As the organism utilizes substrate and converts it into carbon dioxide, metabolic intermediates and other catabolites, NAD+ is reduced to NADH. In aerobic cells, oxygen is used to reoxidize the NADH to NAD+ during oxidative phosphorylation and energy in the form of ATP is produced for use in other metabolic reactions (e.g., growth). Anaerobic organisms operate in a similar manner, but use electron acceptors other than oxygen.

Figure 7-1 may also be used to illustrate the effect of various parameters on the reducing level of the cell - that is, the ratio of NADH concentration to the total NADH + NAD+ concentration in the cell. If in an aerobic system the oxygen supply is limited while there is an adequate supply of substrate present, catabolism will continue to function, but reoxidation of NADH by respiration stops. Under these conditions the reducing level approaches unity. If on the other hand, substrate becomes limiting or depleted, NADH levels fall and the reducing level approaches zero.

By measurement and control of the reducing state of the cell (i.e., the ratio of NADH to total NADH + NAD+), it is possible to maintain the culture in the desired condition for selected growth or metabolic activity.

THE FLUORESCENT SIGNAL

The fluorescent signal received from a fermentation culture is a composite signal which is a function of the number of cells, the reducing state of the cell, and environmental effects. This is illustrated in figure 7-2 for a fed-batch process which may be viewed as a high frequency signal superimposed on a low frequency signal. If the signal is properly decomposed it can reveal information regarding the process. The cell concentration and environmental effects will vary slowly while the metabolic effects (reducing state) due to varying nutrient or oxygen can change rapidly. Decomposition

of the fluorescent signal into its major components can then yield data for process control.

FERMENTOR STUDIES

Figure 7-3 is data obtained during growth of a mammalian cell culture showing fluorescence (NFU values), total cell count (-), percent viability (+), and temperature. Figure 7-4 depicts similar results obtained with a Pediococcus and shows NFU, pH, and viable cell counts (□). In both cases there is a good correlation between cell growth and measured culture fluorescence, but it is also evident that the fluorescent signal has other components - lack of correlation between cell count and fluorescence in early stages of Pediococcus growth was due, for example, to pH effects on cells and background (environmental).

Actual data illustrating how the signal can be decomposed into its specific elements is shown in the next series of figures. Figure 7-5 shows the response of a culture of the yeast Saccharomyces to changes in dissolved oxygen levels. Yeast cells were placed in a nongrowth environment and allowed to metabolize their internal glycogen reserve - yielding a relatively constant fluorescent signal.

At 1.5 hours the air to the fermentor was turned off. As the DO level began to fall and approach zero, there was a step increase in the culture fluorescence signal due to an increase in intracellular NADH. This corresponds to the organism undergoing a metabolic transition from an aerobic to an anaerobic state. It should be noted that the step increase in fluorescence actually occurs prior to a DO reading of zero. This is a consequence of the more rapid response of the FluroMeasure™ Detector relative to a DO probe. By the FluroMeasure™ monitoring intracellular conditions, it indicates that the cell is internally anaerobic before the broth is depleted of oxygen. The air flow was turned back on at 2.0 hours and there was a rapid decrease in the NFU reading back to the level corresponding to aerobic metabolism. After 2.0 hours the culture continued to be in a resting condition. After 2.5 hours there was a rapid decrease in the fluorescence (NFU) signal as the glycogen stores of the yeast became depleted. At approximately 3.0 hours the DO returned to 100% and the level of culture fluorescence (NFU) reached its minimum energy value as metabolism ceased.

In the absence of glucose and glycogen, yeast will aerobically metabolize ethanol via the pathway illustrated in figure 7-6. In this pathway ethanol is metabolized through acetaldehyde to acetate and in both reactions NADH is produced. Acetate is further metabolized through acetyl-CoA into the TCA cycle.

Using high pressure liquid chromatography to monitor the change in extracellular ethanol and acetate concentrations, an ethanol addition cycle was studied and the results are shown in figure 7-7. At 2.4 hours ethanol was added at a concentration of 250 PPM. The ethanol was rapidly absorbed by the cells and its concentration dropped to less than 100 PPM within the first minute after its addition. This was followed by a more gradual decline in the ethanol concentration between 2.5 and 3.0 hours until ethanol was entirely depleted. It should be noted that when the extracellular ethanol concentration is greater than 50 PPM, there is a steady state condition in the NFU signal. As the ethanol concentration drops below this level and approaches the Ks value, it becomes rate limiting and there is a rapid decrease in the level of intracellular NADH. This indicates that the cells are in a transition from one steady state reducing level to another lower reducing level. At 3 hours there is an inflection point corresponding to the exhaustion of ethanol. During this same time period there is an accumulation of acetate in the media. The acetate concentration, which was initially zero, increases to greater than 100 PPM while the ethanol is being utilized. When the ethanol is depleted, acetate re-enters the cell and is then metabolized. Depletion of the acetate corresponds to the next inflection point in the curve at 3.15 hours.

Extending this data to various cell concentrations it is found that a series of curves can be developed relating the NFU value to the active catabolic pathways. This is shown in figure 7-8. The bottom curve, representing the lowest reducing state for the culture, is the NFU values for cells which are in the resting state devoid of metabolizable substrate. The top curve, representing the highest steady state cellular reducing level, is derived from the NFU value for cells converting glucose to ethanol under anaerobic conditions. Similar curves have been generated for aerobic conversion of ethanol to acetate and for acetate metabolism. For any cell concentration, the on-line NFU value can be used to determine the active metabolic pathways. Thus, the reducing state of the cell is a basis for process control.

Fed-batch fermentations with Saccharomyces were conducted using a glucose feed rate based on a known starting biomass concentration and an estimated biomass doubling time. Figure 7-9 shows the NFU and cell mass data for one of these runs. For the first 6 hours after inoculation NFU levels remained constant and then began to rise. Cell mass increased only slightly during this time. The sharp drop off in the NFU value at about 7.5 hours was due to stoppage of the glucose feed. The glucose feed was restored at about 9 hours and both cell mass and NFU readings continued to increase.

For each cell mass value measured in this experiment a corresponding minimum (resting cell) and maximum (glucose to ethanol pathway) reducing value was determined by reference to figure 7-8. Reducing levels for aerobic ethanol utilization were also determined (+-). These values were then plotted along

with the NFU readings from the fed-batch fermentation (fig. 7-9) and are shown in figure 7-10. During production of yeast, 50% of the available glucose theoretically can be converted to cell mass. Production of ethanol results in reduced yields and lower productivity. For optimal yeast cell mass production, therefore, the reducing state should be controlled between the two extreme conditions.

As can be seen from figure 7-10 initial NFU values were high, indicating overfeeding with resulting ethanol production. This was confirmed by off-line HPLC analysis for ethanol. As cell mass began to increase, the NFU reading remained constant and approached a midpoint range between the two extreme values. At about 7.5 hours, stoppage of the feed resulted in an immediate drop in NFU values to the resting cell level. When the feed was restarted at about 9 hours, NFU values increased immediately and then dropped again. From about 11 to 12 hours into the run the NFU values indicated a proper balance between substrate consumption and increase in cell mass. Beginning at about 12 hours; however, an imbalance between these two parameters occurred again resulting in the production of ethanol as evidenced by the increase in NFU values and confirmed by HPLC analysis. A series of oscillations was then noted in which additional ethanol was produced indicative of the Crabtree effect.

From about 15 to 20 hours into the run the glucose feed rate was constant and the cell mass increased to a level where it was no longer being overfed. At this stage the glucose feed was stopped and NFU values dropped and remained at a level corresponding to aerobic ethanol metabolism.

CONCLUSION

These data demonstrate that the measurement of intracellular NADH can be utilized to monitor the internal reducing state of cells. The internal reducing state reflects the major metabolic events which are occurring in the culture. Since these data describe pathways which are common to those found in many procaryotic and eucaryotic organisms, similar information may be used to control a wide variety of fermentations and cell cultures.

It should be possible to develop control algorithms for product induction or feeding strategies to maintain a culture at a selected reducing level corresponding to the desired product output.

REFERENCES

1. Duysens, L. N. M., and Amesa, J., "Fluorescence Spectrophotometry of Reduced Phosphopyridine Nucleotide in Intact Cells in the New-Ultraviolet and Visible Region," Biochem. et Biophys. Acta., 24:19, 1957.

2. Chance, B., "Spectrophotometry of Intracellular Respiratory Pigments," Science, 120:767, 1954.

3. Chance, B., and Thorell, B., "Fluorescence Measurements of Mitochondrial Pyridine Nucleotide in Aerobiosis and Anaerobiosis," Nature, 184:931, 1959.

4. Chance, B., Estabrook, R. W., and Ghosh, A., "Damped Sinusoidal Oscillations of Cytoplasmic Reduced Pyridine Nucleotide in Yeast Cells," PNAS, 51:1244, 1964.

5. Harrison, D. E. F., and Chance, B., "Fluorimetric Technique for Monitoring Changes in the Level of Reduced Nicotinamide Nucleotides in Continuous Culture of Microorganisms," Appl. Microbiol., 19:446, 1970.

6. Zabriskie, D. W., and Humphrey, A. E., "Estimation of Fermentation Biomass Concentration by Measuring Culture Fluorescence," Appl. Environ. Microbiol., 35:337, 1978.

7. Ristroph, D. L., Watteeuw, C. M., Armiger, W. B., and Humphrey, A. E., "Experience in the Use of Culture Fluorescence for Monitoring Fermentations," J. Ferment. Technol., 55:599, 1977.

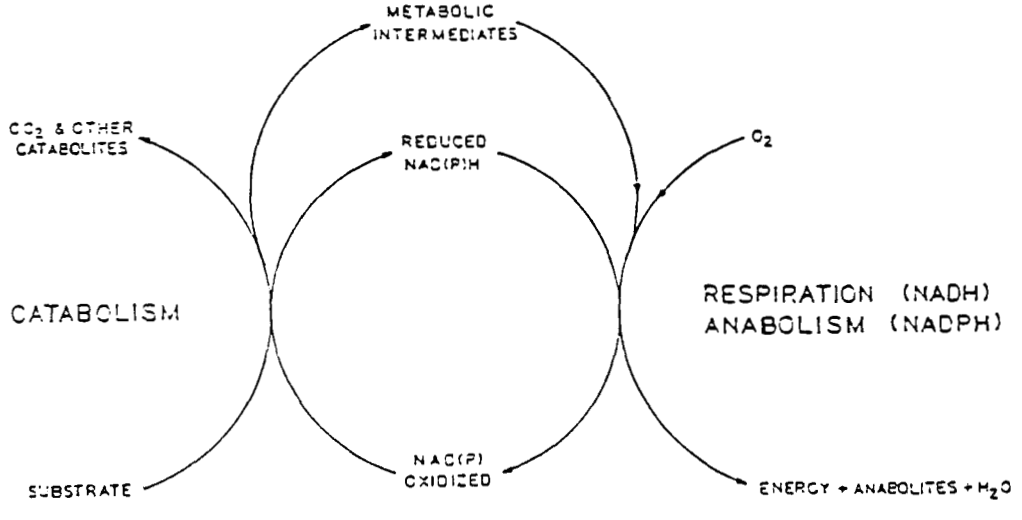

Figure 7-1.- Diagram of basic cellular metabolism showing involvement of oxidized and reduced pyridine nucleotides.

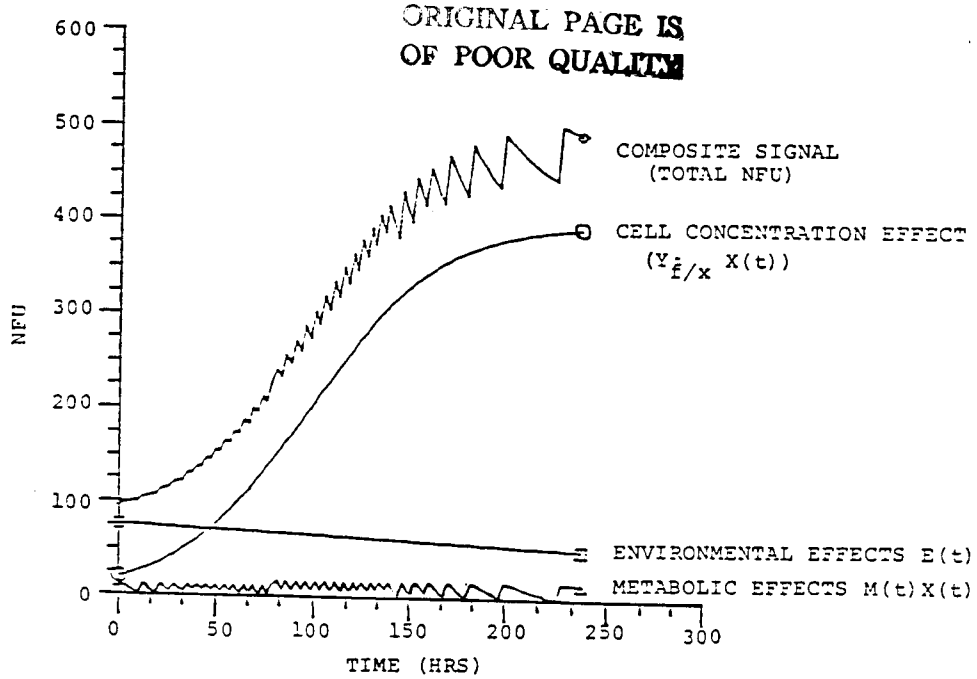

Figure 7-2.- Representation of culture fluorescence as a function of the number of cells, the reducing state of cells, and environmental effects.

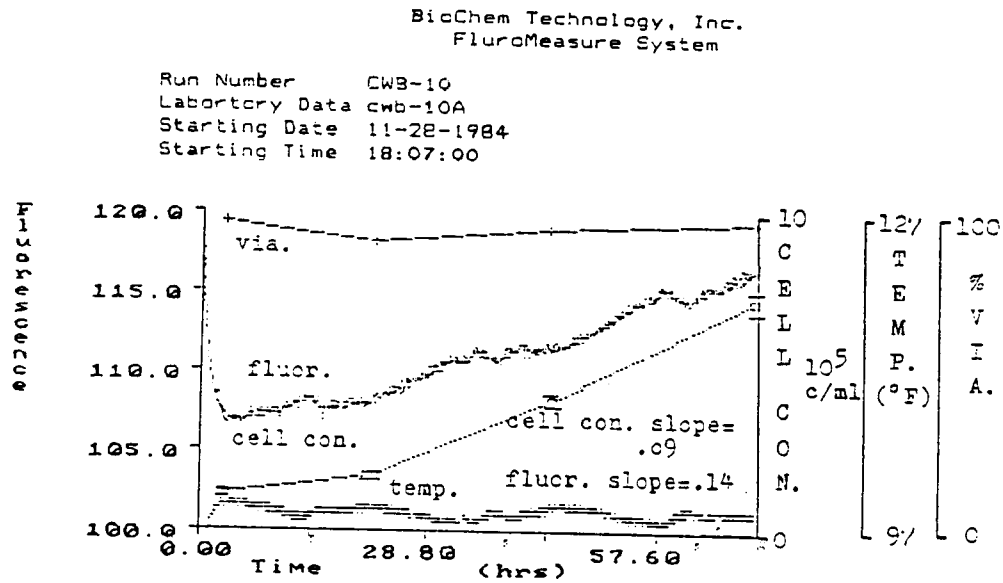

Figure 7-3.- Fluorescent changes during growth of Mouse L29 cells in suspension culture.

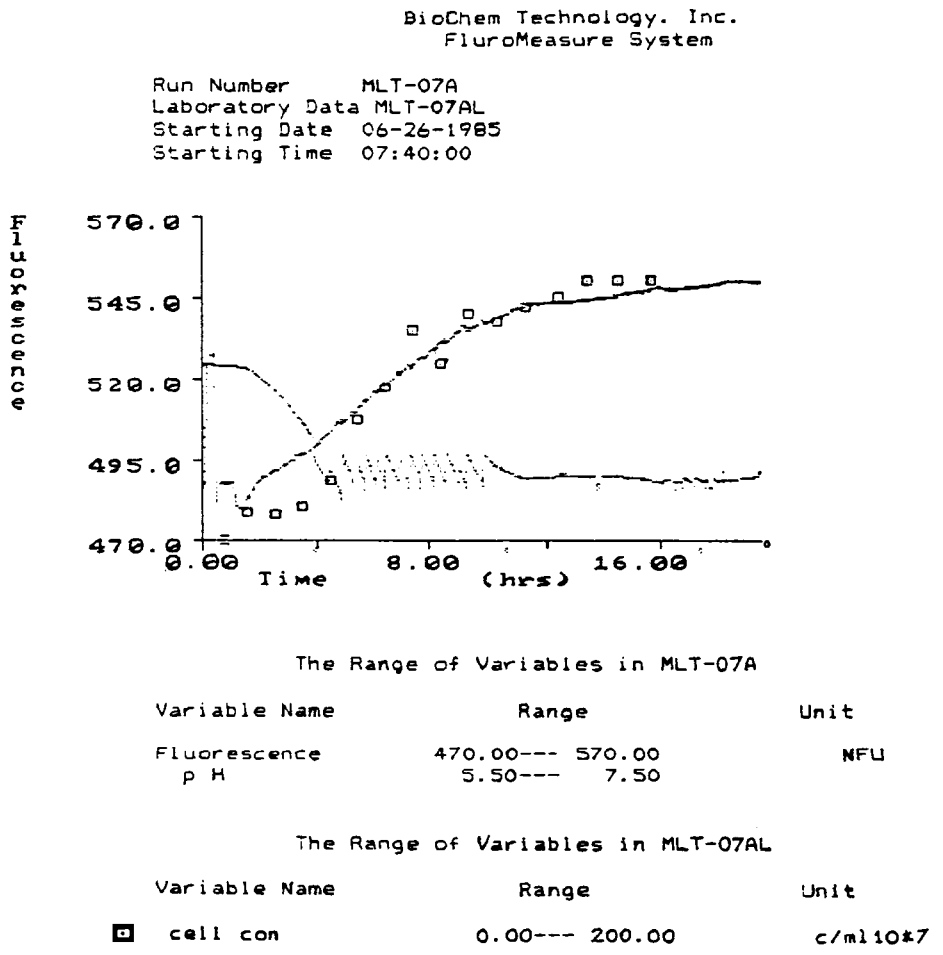

Figure 7-4.- Fluorescent changes during growth of a Pediococcus sp.

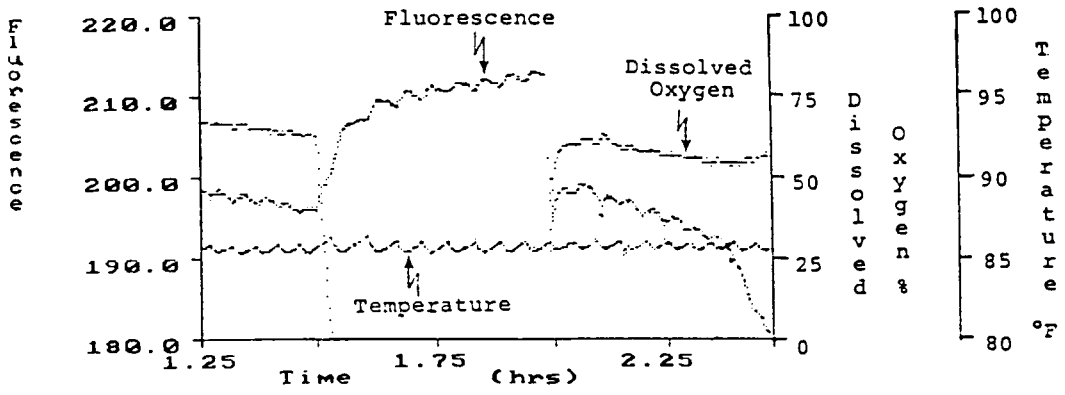

Figure 7-5.- Fluorescent response of Saccharomyces to changes in dissolved oxygen levels.

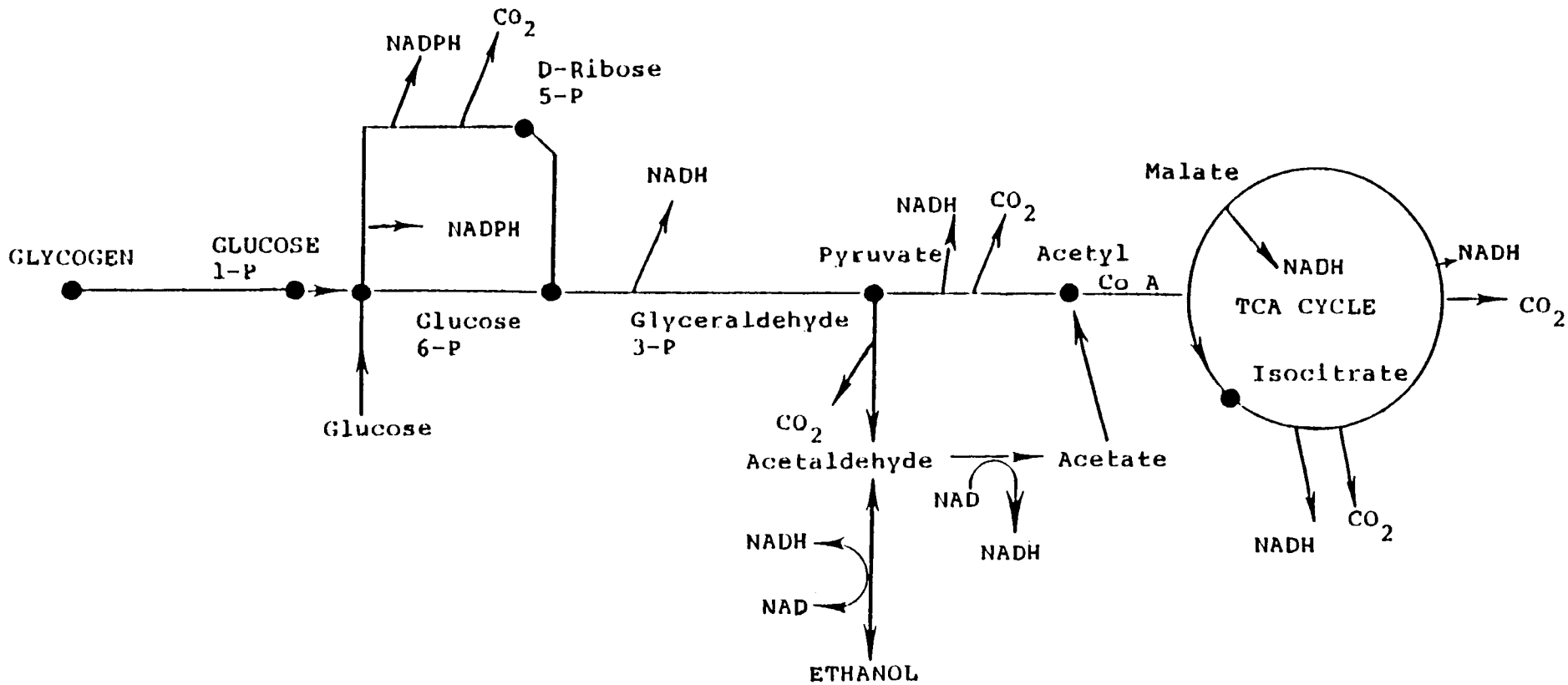

Figure 7-6.- Aerobic ethanol metabolism.

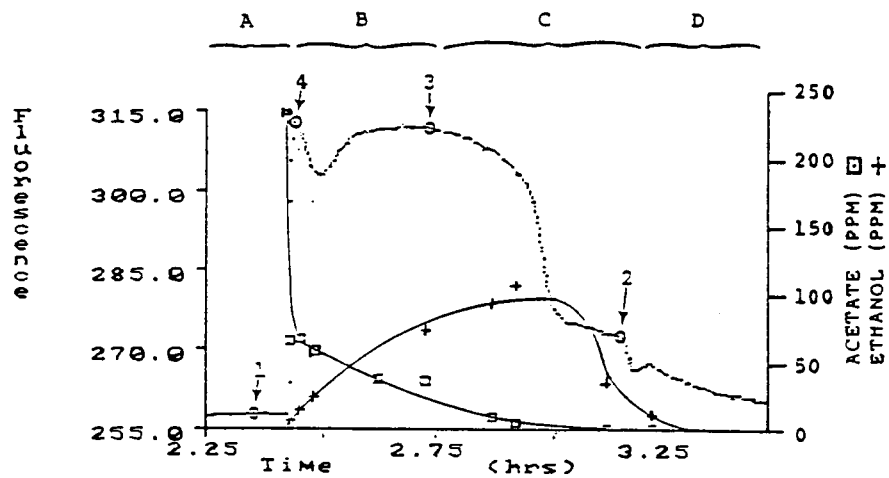

Figure 7-7.- Correlation of fluorescence with ethanol and acetate concentration during ethanol metabolism in starved Saccharomyces.

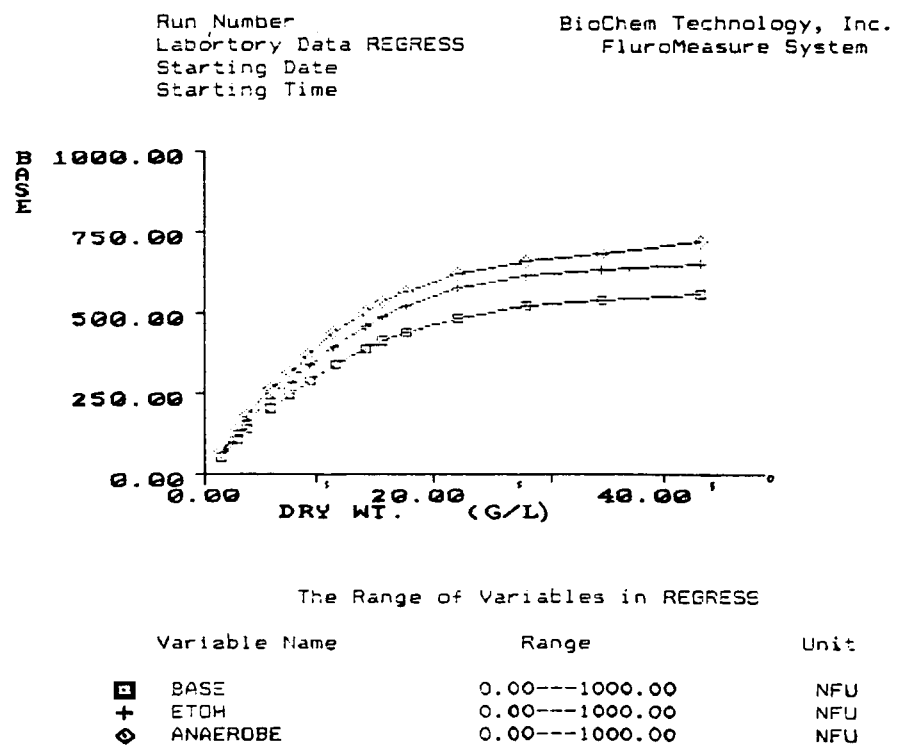

Figure 7-8.- Fluorescence values at various cellular reducing states as a function of dry cell mass.

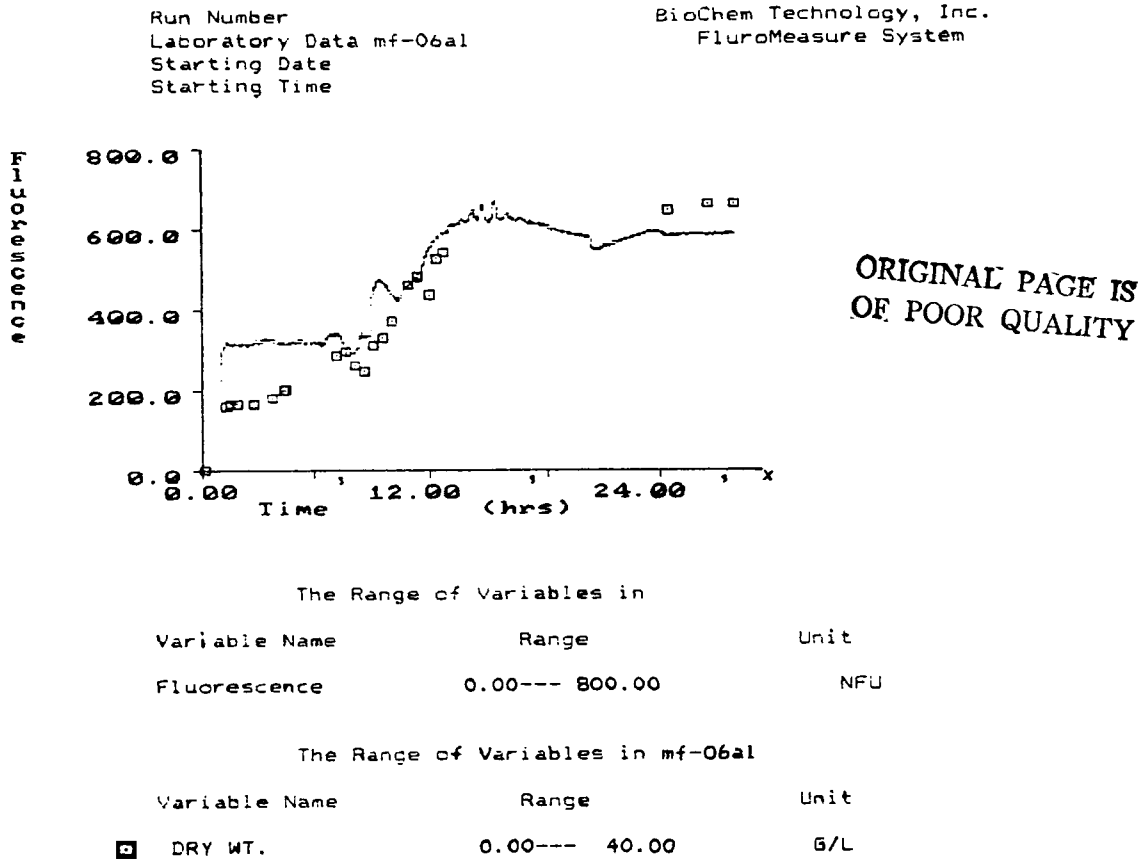

Figure 7-9.- Fluorescence and cell mass changes during fed-batch growth of Saccharomyces on glucose.

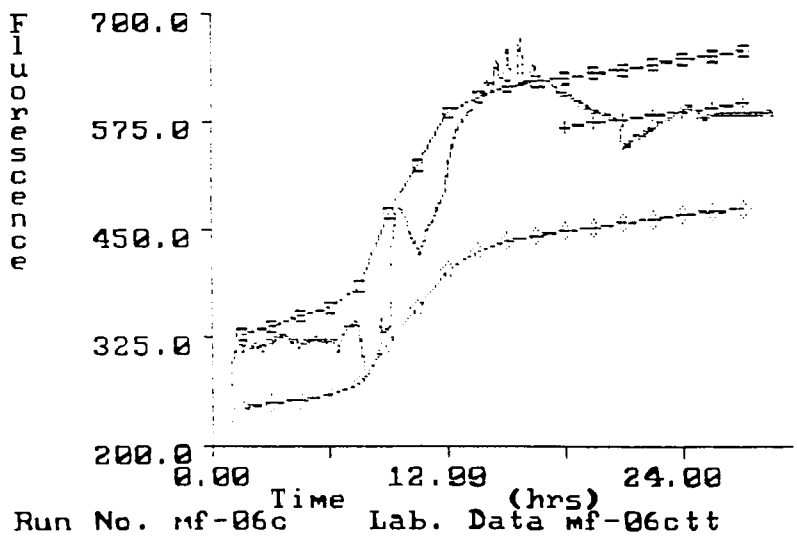

Figure 7-10.- Measured fluorescent values versus theoretical minimum and maximum values for a Saccharomyces fed-batch glucose fermentation.